Final Technical report for FSA project ref G01022

An evaluation of probiotic effects in the human gut: microbial aspects

Prof G.R. Gibson

Dr G. Rouzaud

Food and Microbial Sciences Unit

University of Reading

Prof. J. Brostoff

Dr N. Rayment

King's College London

Executive Summary

Rationale

A variety of probiotic supplements are now available for human use in the UK. These range from fermented milks to lyophilised forms, containing both single and multiple strains. However, there is an almost total absence of comparative data on probiotic effects within the human gut and their inherent safety implications. This project aimed at providing independent information on the effects of existing probiotics in the alimentary tract. The project was designed to address the following issues:

- To compare commercially available probiotic strains characterised and identified in an independent non-FSA funded study.
- To test the survival of probiotics in the gastrointestinal milieu
- To determine whether probiotics affect the composition of the gut microflora

Summary of the approach and objectives

The approach used several validated *in vitro* models simulating the physico-chemical events arising in the stomach, upper intestine and lower intestine. A wide number of strains were isolated from commercial probiotic products and phylogenetically identified. Only probiotic strains originating from products which label matched their microbiological content were chosen for the study. All selected probiotics were treated with digesta resembling the gastric environment. Probiotic strains that were able to survive the stomach conditions were subsequently considered for testing their survival in the upper intestine. This exercise determined which probiotics had the capacity to survive transit to the lower intestine– the target organ for probiotic residence and effects. Survival in the colon was tested in an *in vitro* model of the human large intestine that reflects microbial events in the ascending, transverse and descending regions of the colon. The effect of probiotic on gut microbial balance was also investigated using a molecular methodology.

How closely were the objectives met?

All tasks agreed in the proposal were accomplished. Valuable information on the microbiological effect that probiotics exert were obtained from the study. The project has clarified the most robust/reliable products for gut survival particularly for *Lactobacillus spp.* Further studies would need to clarify the effect of *Bifidobacterium spp.* and *Enterococcus faecium* in the lower intestine.

Outline of the main findings

Microbial strains originating from eight different commercial products were isolated and sequenced. Of the products tested, not all the products displayed a profile of probiotics similar to what was stipulated on their labelling. A collection of 35 bacterial strains was gathered from the initial screening. Principal strains isolated from commercial products were from *Lactobacillus spp*. and *Bifidobacterium spp*.

The survival of thirty two probiotic strains was assessed in simulated gastric contents. After twenty minutes incubation in the presence of simulated gastric contents at pH varying between 1 and 3, *Lactobacilllus spp. ,Bifidobacterium spp. , Enterococcus sp*. and *Lactococcus lactis sp*. showed a good survival in the gastric environment. A ratio was calculated to estimate the proportion of bacterial cells surviving the gastric milieu. Eighteen strains had a ratio of live cells greater than 0.5 at pH 2 or 3 or at both pHs indicating that 50% or more probiotic cells were still viable after 20 minutes in the stomach environment.

On the basis of their resistance to simulated gastric content, the strains selected in the stomach challenge were tested for their tolerance to bile acids. The growth of probiotic strains in MRS broth in the absence or in the presence of bile acid salts was monitored at two concentrations of bile acids 18g.L^{-1} and 36g.L^{-1}. A coefficient of growth inhibition (C_{inh}) was calculated for each bile concentration to characterise the effect of bile acids on probiotic growth. Probiotic strains were classified in three groups according to their coefficient of inhibition. Six strains showed a C_{inh} close to zero indicating little or no effect of the presence of bile salts on the growth of probiotic. Seven strains had their growth slowed down by bile

acids ($0.2<C_{inh}<0.4$). Five strains showed a poor tolerance to bile acid content as shown by a C_{inh} greater than 0.4 and the absence of growth. The strains of the latter group were unlikely to reach the large intestine intact. A sub-set of strains was challenged with simulated upper intestine contents containing pancreatic enzymes. In this test, *Lactobacillus spp*. showed a higher sensitivity to upper intestinal content than predicted from the bile acid tolerance test. On the other hand, *Bifidobacterium sp*. and *Enterococcus faecium* showed a good survival in this environment. *Lactococcus lactis* did not, however, survive well in both assays.

Six Lactobacilllus strains were subsequently selected for test of survival in the lower intestine. The strains studied were *L. casei immunitass, L. casei shirota, L. plantarum, L. pentosus, L. reuterii, L. acidophilus* subsp *johnsonii and L. delbrueckii* subsp *bulgaricus*. Each strain was studied independently. *L. casei shirota, L. plantarum, L. pentosus and L. reuterii* were able to survive in measurable level for five days after inoculation of the strain. *L. casei immunitass, L. delbrueckii* subsp *bulgaricus and L.acidophilus* susp*. johnsonii* displayed poor survival.

Results of the interaction with the residential microflora showed that addition of probiotic did not affect significantly the total number of bacteria growing in the continuous culture. Little difference were also seen on the levels of main bacterial species numerated by Fluorescent *In Situ* Hybridisation. Variability in the composition of the microflora was seen from one gut model run to another but Bacteroides population remained at high levels throughout the 10 days of sampling in all gut models. Bifidobacteria were generally less prevalent at the end of the wash-out period. Although probiotic from *Lactobacillus spp*. were added to the three stage fermentation system, Lactobacillus group remained at sub-dominant level in the three vessels of the fermentor.

Introduction

Background to the project

Dietary modulations of the human gut microflora can be of great benefit to health. The most frequently used dietary method of influencing the gut flora composition is the consumption of probiotics. Over the years many species of micro-organisms have been used. They consist mainly of lactic acid bacteria (lactobacilli, streptococci, enterococci, lactococci and bifidobacteria). *Bacillus spp*. may also be used.

The project aims at comparing the persistence of probiotics within the human gastrointestinal tract. Probiotics are marketed as health or functional foods whereby they are ingested for their purported positive advantages in the digestive tract and/or systemic area. To exert any beneficial properties, probiotics must have robust survival properties in the gut. Given their wide use in the UK, purported health aspect and the fact that they contain living organisms, all new probiotics would be subject to a rigorous pre-market safety evaluation under the terms and conditions of the Novel Foods Regulation (EC) 258/97 before they can be sold in the EU, and all existing probiotic products must comply with the Food Safety Act (1990).

Scientific principle and rationale behind the choice of approach

It is important that reliable and extensive independent data are generated on product survival. Colonisation of the human gut may be prevented by the natural resistance exerted by the commensal gut microflora therefore compromising any effect of the probiotic strain. The work suggested here built on pre-existing information on genetic integrity of commercial products available in the UK markets. Various products have previously been tested for their probiotic content and the isolated strains phylogenetically identified. Products where the probiotic strains matched their label were subjected to a rigorous comparative assessment of effects in the gastrointestinal tract. In vitro approaches were chosen for this study to allow standardisation of testing conditions and direct comparisons between probiotic strains.

Aims and objectives

The various facets that were addressed were: survival in the gastric, small and large intestinal regions and effects on the colonic microflora balance. As extensive studies covering the effect of all existing probiotics in each intestinal regions would be time-consuming, the strategy adopted here was a selective screening. Only probiotic strains showing a positive response in the considered digestive compartment were selected for assessment in the next digestive compartment. Thus, a decrementing number of probiotic organisms was studied at each stage of the project.

Material and Methods

Bacterial isolation of strains from commercial products

Twelve commercial probiotic products were selected from the supermarket shelves and health food shops. One gram of product was homogenised in 9 ml of Phosphate Buffer Saline (PBS, Oxoid, UK). Serial 10-fold dilutions were subsequently prepared in anoxic diluents. Diluents contained half strength peptone water (7.5 $g.L^{-1}$, Oxoid, UK) and L-cysteine HCL (0.5 $g.L^{-1}$, BDH, UK) adjusted at pH 7.0. Selected dilutions (10^{-2} to 10^{-6}) were plated in duplicate on four growth media: Wilkins-Chalgren (WC) agar (Oxoid, UK), Raffinose Bifidobacterium agar (Hartemink *et al*, 1996), Beerens agar (Beerens *et al*, 1996), Rogosa agar (Oxoid, UK). Plates were incubated anaerobically for 48h at 37ºC. Colonies with different phenotypes were selected and sub-cultured on WC agar plates until pure strains were obtained. Isolated strains were maintained on cryogenic beads in Microbank tubes at -70ºC. For later experiments, probiotic strains were revived on Mann, Rogosa and Sharp agar (MRS agar, Oxoid, UK) and sub-cultured on MRS broth (Oxoid, UK) in anaerobic conditions.

Bacterial characterisation by genotyping prior to this project starting

Isolated probiotic strains were identified by amplifying and sequencing the hyper-variable regions of the bacterial 16S rRNA. The nucleotidic sequence obtained was compared to sequences stored in databases to ascertain identity.

DNA extraction and phylogeny

Isolates were revived on Wilkins-Chalgren anaerobes agar. DNA was extracted using a phenol/chloroform technique (Lawson *et al.*, 1989). 16s rRNA gene was amplified by 34 cycles Polymerase Chain Reaction (PCR) using universal 16s rRNA gene forward primer pA (Eden *et al*., 1991) and reverse primer pE (Edwards *et al*., 1989).

PCR amplification was carried out using PCR AmpiTaq® kit. PCR cycling was performed on a Gene Amp PCR system 9600 thermocycler (Cetus, Perkin-Elmer). A QIA-quick PCR purification kit (QIAgen Ltd, West Sussex, UK) was used for purification of the DNA strands.

A portion of the PCR product proximal to the 5' end of the rRNA was sequenced (~ 500 nucleotides). ABI PRISM™ dRhodamine terminator cycle sequencing kit with the AmpliTaq DNA polymerase FS (PE Applied Biosystems Inc., California) and an automatic DNA sequencer (model 373A; PE Applied Biosystmes Inc.) were used. Initial screening of the isolates was carried out using reverse primers pD* and δ (Lawson *et al.*, 1989)
Sequences generated were compared to bank collection using FASTA. Identification was determined on the basis of >97% homology to the most closely related sequence.

The output from this earlier study was required to select appropriate commercial strains. The initial objective was therefore "**Identify products to be tested"** and Task 01 was "**Selection of probiotic products".**

Survival in simulated gastric content

Overnight culture of probiotics were submitted to pH values ranging from 1- 3. A control treatment was run at pH 6.5. Pepsin (Sigma, UK) was added to the experimental medium at all pHs to simulate the activity of gastric enzyme. The number of live probiotic cells was assessed by plating out tested suspensions on MRS agar at T0 and 20 min. Simulated gastric juice (SCJ) medium was made of peptone water (7.5 $g.L^{-1}$) dissolved in distilled water and adjusted at pH 1, 2, 3 or 6.5 using 1 M Hydrochloric acid (BDH, UK). SCJ medium was aliquoted in Hungate tubes (9.5 ml per tubes), reduced anaerobically and autoclaved. Prior to the experiment, a fresh solution of pepsin (300 $mg.L^{-1}$) was made up in 0.5 ml simulated gastric juice, incubated at 37°C for 5 min and added to Hungate tubes (final concentration 15 $mg.L^{-1}$). Pure cultures of probiotics were grown anaerobically overnight in 10 ml of MRS broth. The inoculum was concentrated by centrifugation at 2000 rpm. The pellet was re-suspended in 1 ml of SCJ taken from Hungate tubes, the mixture was immediately transferred in the corresponding Hungate tubes. At T 0 and 20 min, one millilitre sample was taken and diluted serially eight times in peptone water (14 $g.L^{-1}$, pH 6.5). Dilutions 1 to 8 were plated out on WC agar in duplicates. Plates were incubated for 48h in anaerobic cabinet at 37°C before numeration. To compare bacterial survival, a ratio was calculated as follows:

r = Average of cells at T20 / Average of cells at T0.

If r=1, bacterial survival was not affected by the simulated gastric environment. A ratio equal to 0.5 indicated a loss of half the viable cells present in the culture. A ratio greater than one indicated bacterial growth. The threshold for selection of bacterial strains for subsequent tests was fixed at r=0.5.

Bile acid tolerance

The rapidity of growth in a broth medium with and without bile acids was assessed using a validated method (Charteris *et al*., 1998). One millilitre of overnight culture was inoculated in Hungate tubes containing 9 ml MRS broth supplemented with 18 $g.L^{-1}$ or 36 $g.L^{-1}$ oxgall (Sigma, UK). Non-supplemented MRS broth was used as control treatment. Hungate tubes were incubated anaerobically at 37°C for 8 hours. Cultures were monitored hourly for growth

by measuring absorbance of the culture medium at 650 nm. Coefficient of inhibition was calculated using a method adapted from Gopal *et al.* (2001) as follows:

$$C_{inh} = (\Delta_{T8-T0}\,\text{Control} - \Delta_{T8-T0}\,\text{Treatment})/(\Delta_{T8-T0}\,\text{Control})$$

Where ? represented the difference in absorbance between T0 and T8. The experiment was done in triplicate. Only strains achieving a C_{inh} smaller than 0.40 at both concentrations of bile acids were considered for further experiments.

Survival in simulated upper intestine content

The survival of probiotic strains to simulated upper intestine content was achieved by subjecting the probiotic cells to a micro-aerophilic alkaline medium containing bile salts and a mixture of pancreatic enzymes. The survivability of the cells was measured before and after 2 hours of incubation at 37°C. Simulated upper intestine juice (UIJ) consisted in Bile salts no 3 (1 $g.L^{-1}$, Oxoid, UK) suspended in 1M PBS solution (pH 8). UIJ was aliquoted in Hungate tubes (9ml per tube). Prior to the experiment, a freshly prepared solution of Pancrex (final concentration 1 $g.L^{-1}$, Paines and Byrne Ltd, UK) was added to Hungate tubes and incubated anaerobically at 37°C for 5 min. One millilitre of overnight culture was subsequently inoculated. At T 0 and 2 hours, one millilitre sample was taken and diluted serially eight times in half-strength peptone as described previously. Dilutions 1 to 8 were plated out on MRS agar in triplicates. Plates were incubated for 48h in anaerobic cabinet at 37°C before numeration.

Survival in simulated large intestine

A validated model of the human colon reflecting the physico-chemical conditions in the proximal, transverse and distal regions of the colon was used to determine the survival of probiotic strains in mixed culture environment (Macfarlane *et al*., 1998).

Gut model system

The system consists in three vessels V1, V2 and V3 arranged in series, with respective working volume of 0.28 L, 0.30 L and 0.30 L. Temperature was maintained at 37°C and the pH was automatically controlled to maintain values of 5.5 (V1), 6.2 (V2) and 6.8 (V3). All

vessels were kept under a headspace of oxygen-free nitrogen gas (0.9 $L.h^{-1}$) and continuously stirred. The culture medium feeding the system was reflecting the components entering the proximal colon and was prepared according to validated protocol (Macfarlane *et al.*, 1998). The culture medium was kept sterile and anoxic by sparging O_2-free N_2. Medium was fed to V1 by a peristaltic pump which sequentially supplied V2 and V3. Flow rate was set to obtain a final retention time of 36 hours throughout the model.

Preparation of inoculum

Vessels were sterilised and filled with autoclaved feeding medium via the peristaltic pump. To maintain anaerobiosis oxygen-free nitrogen gas was sparged in the system for 12 hours prior to inoculation with faecal microflora. Inoculum consisted in 100ml of faecal slurry from a healthy human donor. The slurry was prepared by homogenizing freshly collected faeces in anoxic PBS at pH 7.2 (final concentration 200 $g.L^{-1}$). The cultures were allowed to equilibrate overnight before fresh medium was added to the system.

Experimental design and sampling protocol

Gut Model was run until equilibration was reached. Stability period was determined when seven turnovers of the feeding medium have run through the complete system. When steady state conditions were reached, a total of 4 ml of a washed probiotic preparation was added daily to V1 for a period of five consecutive day (Day 1 to Day 5). From Day 6 to day 10, the system was run with no probiotic addition to measure the survival of probiotic in the mixed culture environment. This experimental design was adopted to simulate the daily human consumption of probiotic product. Five millilitres of samples were taken from each vessel daily for analysis of bacterial composition.

Preparation of probiotic strain for inoculation

To differentiate the probiotic strain fed to the multi-stage gut model from similar bacterial species present in the indigenous microflora, rifampicin-resistant mutant of the studied strains were isolated. The resistant variant of probiotic were selected by growing successive overnight, anaerobic cultures in MRS broth containing increasing amount of rifampicin (0.100

to 100 µg.L^{-1}) until growth was observed in the medium with the highest antibiotic concentration. Rifampicin–resistant strains were isolated on MRS agars supplemented with rifampicin (100 µg.L$^{-1,}$ sigma, UK) and kept on cryogenic beads in Microbank tubes at -70ºC. Prior to the study, overnight cultures (100ml) of the probiotic strain were grown in MRS broth supplemented with rifampicin (100 µg.L^{-1}) under anaerobic condition. Cells were centrifuged at 3000 rpm (5 min) and washed in 20 ml 1M PBS (pH 7.2) twice. The final pellet was re-suspended in 5 ml of feeding medium. One millilitre of concentrated probiotic was kept for numeration while the remaining 4 ml were added to V1.

Numeration of probiotic cells in the gut model

The concentration of probiotic inoculated to the model as well as the survival of probiotic in vessels V1, V2 and V3 was numerated on MRS agar supplemented with rifampicin (100 µg.L^{-1}) after serial dilution in anoxic half strength peptone water. Dilution 1 to 8 were plated in duplicate and anaerobically incubated for 72h prior to numeration.

Analysis of bacterial composition

Fluorescent *in situ* hybridisation was used to numerate the predominant gut genera present in the gut model (Harmsen *et al*, 1999). Genus specific 16S rRNA-targeted oligonucleotide probes labelled with the fluorescent dye Cy 3 were used for enumerating *Bifidobacterium, Bacteroides, Lactobacillus/Enterococcus* and *Clostridium* subgroup *histolyticum bacteria* . Samples (375 µl) were fixed with filtered paraformaldehyde (1125µl of a 40 g.L^{-1} solution). The remaining procedure was identical to that described by Rycroft *et al*, 2001. Total number of bacterial cells was assessed using the nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis

Statistical analysis was performed using two-tailed Student's t-test, assuming equal variance between two samples. Statistical significance was $p<0.05$.

Results and discussion

Prior to the commencement of this study, a total of thirty five bacterial strains were isolated and identified from commercial products. The identity of the bacterial strains and their subsequent use in the series of tests is summarised in Table 1. Strains included 19 isolates of *Lactobacillus spp*., 5 isolates of *Bifidobacterium spp*. One isolate of *Lactococcus lactis sp*. and 5 isolates of *Enterococcus sp.* The sources of each of these probiotic strains were from products available in UK supermarkets and health stores as summarised in Table 2. Commercial products were chosen to be representative of the formulation currently available on the market. These comprised dairy products, yoghurts and fruit juices containing live bacteria or dry preparations in the form of capsule, tablets or powder.

Survival in simulated stomach contents

The typical transit time of a small food bolus in the stomach is approximately 20 minutes. This period of time was therefore chosen to test the resistance of bacterial strains to simulated gastric contents. Stomach acidity varies according to individuals and whether individuals has fasted prior to ingestion. To account for these inter-individual differences, the ability of probiotic strains to survive at various acidic pHs was investigated. The effects of simulated gastric digestion on 32 strains from our collection are presented in Table 3. *Lactobacillus casei* (isolate BeIi2), *Lactobacillus plantarum* (isolate BeH1), *Enterococcus faecium* (isolate RbC2) could not be revived adequately on WC agar and were not included in the selection. At pH 1, 17 strains did not survive the stringent acidity of the simulated gastric medium immediately after inoculation as shown by the low recovery of cells at T0 for isolates BeC1, BeC2, BeG1, BeIi1, RbC1, RbIi1, RbIi2, Strain 46, Strain 47, WCA1, WCA3, WCC1, WCD1, WCF1, WCG2, WCG3, WCH1. Acidic stress may have repress cell growth and may explain the absence of colonies on agar plates. Some strains were able to recover after 20 min such as *Lactobacillus pentosus* WCA2. Stress due to pH acidity and subsequent recovery effects were observed to a lesser extent at pH 2 and 3. These events highlighted the variability in survival rate that has been reported *in vivo*. Strains were assessed according to their survival rate as determined by the ratio of live cells T20 to T0. Among the strains most able to survive gastric acid conditions, eight strains showed more than 50% survival at pH 2 and 3. They

were *Bifidobacterium sp.* (RbG1), *L. acidophilus* strain JVT5 (RgC2), *L. reuterii* (strain 48), *L. pentosus* (WCA2), *E. faecium* (WCB1), *L. delbrueckii* subsp. *bulgaricus* strain JHWW5 (WCF1), *L. plantarum* (WCG1), *L. acidophilus* subsp *johnsonii (WCG2)*. Additionally, *L. acidophilus* strain JVT5 (BeA1) and *L. plantarum* (RgH1 and WCH1) were amongst the most resistant strains at pH 2. Finally seven strains displayed good survival at pH 3. They were *Bifidobacterium longum* (RbIi2), *L. pentosus* (RgA1), *L. casei immunitass* (strain 46), *L. casei shirota* (strain 47), *L. delbrueckii* subsp. *bulgaricus* strain JHWW5 (WCA3), *Lactococcus lactis* subsp. *lactis* IL1403 (WCC1) and *Bifidobacterium lactis* (WCC2). Strains resistant to pH 2 or 3 were likely to reach the upper intestine intact, they were thus selected for subsequent experiments on bile acid tolerance and resistance to upper intestine environment.

Bile acid tolerance

The tolerance to bile acid was determined in increasing concentration of bile salts according to Walker and Gililand's procedure (1993). Data obtained are summarised in Table 4. *Lactobacillus plantarum* (BeH1, WCG1 and WCH1) and *L. pentosus* (RGA1 and WCA2) were the most tolerant strains in the tested conditions. Larger differences were observed between different isolates of the same strain such as *L. delbrueckii* subsp *bulgaricus* (WCF1 and WCA3) and *L. acidophilus strain* JVT5 (BeA1 and RGC2). Generally the coefficient of inhibition was significantly lower at the highest concentration of bile, illustrating a negative effect of bile secretion in the upper intestine on the survival of probiotic cells. *Bifidobacterium lactis* (WCC2) and *Lactococcus lactis* subsp*. lactis* IL1403 showed poor growth in the control treatment which made the assessment of tolerance to bile acids difficult for these specific strains. MRS substrate and anaerobic conditions used in this experiment may not be suitable for assessment of *Bifidobacterium spp*. and *Lactococcus spp*.

Survival in upper intestine content

A method developed by Charteris *et al*. (1998) was used to determine the survival of probiotics in the presence of bile salts, pancreatic enzymes and alkaline pH. The effect of simulated intestine contents on probiotic strains is summarised in Table 5. *Enterococcus faecium* (RgC1), *Bifidobacterium lactis* (RbC1 and WCC2) were the least sensitive strains to

simulated upper intestine contents. *Lactococcus lactis* subsp *lactis* also showed good survival in this test. The most sensitive probiotic strains were from *L. casei* and *L. acidophilus* group. Results contrasted from those obtained with Walker and Gililand's test. In the present experiment, facultative anaerobic bacteria displayed better survival than organism requiring anaerobiosis for optimal growth. The alkaline conditions (pH 8) may have also compromised the survival of the most acidophilic bacteria present in our collection. Greater sensitivity of *Lactobacilli spp.* to simulated intestine content have been reported in other studies (Charteris *et al*. 1998). The presence of milk protein or mucin was shown to increase survival of probiotic in the upper intestine contents (Fernandez et al, 2003; Charteris et al, 1998). In our study, most of the probiotic strains were presented to the consumers in the form of dairy products or enteric coated capsules. Milk protein and enteric coating may limit the deleterious effect of upper intestine contents when these product are ingested.

Survival in simulated large bowel content

Survival in the large bowel content was assessed for Lactobacilli showing the strongest acid and bile tolerance. Seven Lactobacillus strains were selected: *L. casei immunitass* (strain 46)*, L. casei shirota* (strain 47*), L. plantarum* (WCH1*), L. pentosus (WCA2), L. reuterii* (strain 48*), L. acidophilus* subsp. *johnsonii (*WCG2) *and L. delbrueckii* subsp. *bulgaricus* (WCF1). Rifampicin-resistant mutants were obtained to discriminate probiotic strains from similar species present in the residential microflora of the three-stage continuous system. Rifampicin-resistance was obtained by chromosomal genetic alteration thereby preventing distribution of the rifampicin-resistance among the residential microflora. Concentrations of probiotic strain present in each compartment of the three-stage system are summarised in Table 6. In the inoculum, probiotic concentrations showed variability between strains. Inocula from *Lactobacillus plantarum* and *Lactobacillus pentosus* contained a concentration of live cells in the range of 10^{10} CFU/ml whereas inocula from *Lactobacillus reuterii, Lactobacillus acidophilus* subsp. *johnsonii and Lactobacillus delbrueckii* subsp. *bulgaricus* contained on average 10^5 to 10^6 CFU/ml. *Lactobacillus immunitass* was fastidious to grow in the chosen conditions with no live cell surviving in the inoculum at day 2, 4 and 5. Ideally a yield of 10^7 CFU/ml was expected and seed cultures were incubated until corresponding absorbance was

reached. A greater sensitivity to the centrifugation and concentration procedure may have led to the low survival observed for *Lactobacillus acidophilus* subsp. *johnsonii, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus immunitass*.

During the inoculation period (Day 1 to Day 5), probiotics were found in higher concentrations in Vessel 1. The acidic conditions and availability of substrates in vessel 1 may explain a higher level of colonisation of this vessel. However *L. reuterii* colonised primarily vessel 2 whereas *L. acidophilus* subsp. *johnsonii* was present at its highest level in vessel 3. When probiotic inoculations were stopped, colonisation profile changed for all probiotic strains although no statistical difference was found between the concentrations of cells present during the inoculation period and the wash-out period in all vessels (Table 6). Due to poor numbers of cells in the inoculum, *L. casei immunitass* did not survive in the three-stage fermentation system (Figure 1). *L. shirota* was established at its higher level in vessel 2 at Day 6 (Figure 2). When the addition of fresh inoculum was stopped (Day 5), a decrease of the population levels were observed in vessel 2 and 3. Level of colonisation in vessel 1 remained stable at 10^6 cells/ml throughout the wash-out period suggesting that *L. casei shirota* could potentially survive and compete with the residential gut microflora. *L. plantarum* successfully colonised the three vessels of the fermentation system with the highest concentration in vessel 1. After five days of inoculation, the strain appeared to be persisting in the gut model and able to maintain a substantial population in the range of 10^6 cells/ml (Figure 3). Of all the strains studied, *L. plantarum* showed the highest colonisation in the three stage fermentation system. *Lactobacillus pentosus* demonstrated a similar profile to *L. plantarum*, with a rapid colonisation of the three fermentation vessels (Figure 4). The concentration was the highest in vessel 1 at Day 4. From Day 5 *L. pentosus* gradually decreased to a residual level of 10^4 cells/ml. *L. reuterii* was able to survive in the gut model system but the level of colonisation was lower than *L. plantarum* and *L. pentosus* (Figure 5). Daily variations were found in the number of probiotic cells recovered in vessel 1. *L. reuterii* showed preferential growth in vessel 2 and 3 but strain survival declined in the last days of the wash-out periods (Day 8, 9 and 10) suggesting a transient colonisation of *L. reuterii* in the large bowel when the strain was not fed continuously. L. *acidophilus* subsp. *johnsonii* showed a delayed colonisation of the fermentation vessels due to the low concentrations of cells in the inocula. (Figure 6).

Vessel 2 had on average the highest colonisation. A residual number of probiotic cells were persisting at day 10. A delay in colonisation was also observed for *L. delbrueckii* subsp. *bulgaricus* (Figure 7). Although the strain was present at lower level than other probiotic strain in the inoculation period, the numbers of cells increased over the wash out period to reach a final concentration greater than those observed for *L. reuterii* and *L. acidophilus* subsp. *johnsonii.*

Overall *L. plantarum* showed the best ability to colonise the three compartment of the lower digestive tract for the period of the study followed by *L. pentosus, L. casei shirota* and L*. reuterii*. The survival of *L. casei shirota* and *L. reuterii* have already been documented *in vivo* (Yuki *et al*., 1999; Casas *et al*., 2000). Generally strains coming from human sources such as *L. plantarum and L reuterii* were most able to survive in the lower digestive tract than dairy strains such as *L. acidophilus* subsp. *johnsonii* and *L. delbrueckii* subsp. *bulgaricus.* A preferential colonisation of Vessel 1 may be explained by the large availability of carbohydrates for fermentation. The maintenance of an acidic pH may also be beneficial for Lactobacilli strains. The ability of probiotic strains to survive in Vessel 2 and 3 where the amount of carbohydrates is reduced is beneficial for the host. These probiotic bacteria may maintain fermentative activities in the distal part of the gut thereby preventing the release of deleterious breakdown products from bacterial proteolytic activities.

Influence of probiotics on the composition of the gut microflora

Total number of cells in vessel 1, 2 and 3 was in the range of 10^9 bacteria /ml of gut model medium (Table 7) which is in good correlation to levels found in the bowel *in vivo* (Macfarlane *et al.,* 1998). Overall, little variation of the total number of cells present in the three different vessels were observed between Day 1 and Day 10, although a substantial number of probiotic bacteria were added daily. This is in accordance with the bacterial homeostasis found in the gut. In this experiment, four major groups of intestinal bacteria were quantified to determine whether probiotics influenced the microbial composition of the indigenous microflora. Bacteroides were numerically predominant populations and numbers were generally inversely correlated with the presence of bifidobacteria. There was a trend to a

decrease of bifidobacterial group between Day 1 and Day 10. The addition of probiotic from the lactobacilli group did not contribute to the variation in this population.

Conclusions

- Summary of main findings

The project has generated a whole data set on the ability of commercial strains to survive *in vitro* conditions that mimic individual physiological environments of the intestinal tract. The choice of *in vitro* systems allowed direct comparison between bacterial strains. A procedure difficult to achieve in *in vivo* experiments. The collection of strains used in this project covers most of the probiotic bacteria referenced in the literature and therefore can also be relevant to other commercial products that were not tested during the course of the project. Lower intestinal challenge data are not currently available in the literature. These data constitute important information and material for publication.

- How closely objectives were met

Many probiotic products are available and our data indicates that the products that are most known from the consumer were found to match their content in quality and content. Not all bacterial strains used as probiotics have the ability to survive the intestinal digestion. Overall the study showed that *Lactobacillus spp.* were resistant to the gastric environment but were sensitive to upper intestinal content. Conversely, *Bifidobacterium spp*. were more likely to be affected by stomach digesta but survived well in the upper intestine compartment. The project enabled the consolidation of research methodologies available for determination of probiotic functionality. This approach brought information on probiotic survival limitations and survival in the gut.

- Limitations in the approach taken

Probiotic strains were studied individually in standardised conditions that may not have been optimum for their specific growth. Commercial products are diverse and some may contain a mixture of several strains. Interaction between probiotic strains may enhance survival and the biological effect. For instance probiotic VSL#3 containing high concentrations of several probiotic strains was shown to enhance the colonisation of the intestinal environment by ingested probiotic bacteria (Brigidi *et al.*, 2003). Similarly the presence of milk protein and specific substrates such as lactose or other oligosaccharides in commercial probiotic products may optimise the survival of probiotic strains. The successful improvement of probiotic

survival in synbiotic products – products combining probiotic strains and resistant carbohydrates - has been demonstrated for *Lactobacillus* and *Bifidobacterium spp*.(De Boever *et al*, 2001; Desmond *et al*, 2002; Crittenden *et al*., 2001; Wang *et al*., 1999) Moreover, the intake of a food bolus at the time of probiotic ingestion may also affect the survival of probiotic bacteria. These changes were accounted for to a limited level in the gastric and upper intestine contents simulation where varying pHs and bile concentrations were tested. In our approach however, bacteria were sub-cultured on preferential growth agar prior to testing. The integrated course of event from storage to passage in the different gut compartments was not represented fully in this study and would need further investigation. Finally, survival of probiotic strain through the digestive tract does not systematically lead to beneficial response from the host. The combination of digestive bacterial homeostasis and mature immune system in healthy subjects may mask the potential probiotic effect. The effect may however be effective in populations where the immune system is immature or weak such as infants or the elderly. A thorough *in vivo* investigation of the probiotic strains included in this study as well as more information on the impact of probiotics on the immune system would be required to achieve a comprehensive overview of the efficacy of probiotics in human.

References

Beerens H (1990) An elective and selective isolation medium for *Bifidobacterium spp. Letters in Applied Microbiology* **11**, 155-157.

Brigidi P, Swennen E, Vitali B, Rossi M, Matteuzzi D (2003) PCR detection of Bifidobacterium strains and *Streptococcus thermophilus* in feces of humans subjects after oral bacteriotherapy and yogurt consumption. *International Journal of Food Microbiology* **81**, 203-209

Casas I, A. & Dobrogosz W, J. (2000) Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. *Microbial Ecology in Health and Disease* **12**, 247-285.

Charteris WP, Kelly PM, Morelli L & Collins JK (1998) Development and application of *in vitro* methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. *Journal of Applied Microbiology* **84**, 759-768.

Crittenden RG, Morris LF, Harvey ML, Tran LT, Mitchell HL, Playne MJ (2001) Selection of a Bifidobacterium strain to complement resistant starch in a symbiotic yoghurt. *Journal of Applied Microbiology* **90**, 268-278

Eden PA, Schmidt TM, Blakemore RP & Pace NR (1991) Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *International Journal of Systematic Bacteriology* **41**, 325

De Boever P, Wouters R, Verstraete W (2001) Combined use of Lactobacillus reuteri and soygerm powder as food supplement. *Letters in Applied Microbiology* **33**, 420-424

Desmond C, Ross RP, O'Callaghan E, Fitzgerald G, Stanton C (2002) Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. *Journal of Applied Microbiology* **93**, 1000-1011

Edwards U, Rogall T, Emde M, Bootger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acid Research.* **17**, 7843-7850

Gopal PK, Prasad J, Smart J & Gill HS (2001). *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *International Journal of Food Microbiology* **67**, 207-216

Harmsen H.J.M., Elfferich P, Schut F & Welling G (1999) A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridisation. *Microbial Ecology in Health and Disease* **11**, 3-12.

Hartemink R, Kok BJ, Weenk GH & Rombouts FM (1996) Raffinose-Bifidobacterium (RB) agar, a new selective medium for bifidobacteria. *Journal of Microbiological Methods* **27**, 33-43

Lawson P (1989). Recognition of *Fusobacterium nucleatum* subgroups Fn-1, Fn-2 and Fn-3 by ribosomal RNA gene restriction patterns. *FEMS Microbiology Letters*. **53**, 41-45.

Macfarlane G, T., Macfarlane S & Gibson G, R. (1998) Validation of a three stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial Ecology in Health and Disease* **35**, 180-187.

Rycroft C, E., Jones MR, Gibson G, R. & Rastall R, A. (2001) Fermentation properties of gentio-oligosaccharides. *Letters in Applied Microbiology* **32**, 156-161.

Walker DR & Gilliland SE (1993) Relationship among bile tolerance, bile salt deconjugation, and assimilation of cholesterol by *Lactobacillus acidophilus*. *Journal of Dairy Science* **76**, 956-961.

Wang X, Brown IL, Evans AJ, Conway PL (1999) The protective effects of high amylose maize (amylomaize) starch granules on the survival of Bifidobacterium spp. In the mouse intestinal tract. Journal of Applied Microbiology **87**, 631-639.

Yuki N, Watanabe K, Mike A, Tagami Y, Tanaka R, Ohwaki M & Morotomi M (1999) Survival of a probiotic, *Lactobacillus casei* strain *Shirota*, in the gastrointestinal tract: selective isolation from faeces and identification using monoclonal antibodies. *International Journal of Food Microbiology* **48**, 51-57.